# α-Tocopherol-Induced Hexagonal $H_{II}$ Phase Formation in Egg Yolk Phosphatidylcholine Membranes

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**The effect of α-tocopherol and its acetate on the membrane structure of egg yolk phosphatidylcholine (egg PC) dispersions was investigated using phosphate-31 nuclear magnetic resonance ($^{31}$P-NMR) and small-angle X-ray diffraction. The incorporation of α-tocopherol into egg PC dispersions induced a change in the $^{31}$P-NMR spectrum from a multilamellar bilayer line shape to a hexagonal $H_{II}$ one. The phase transition by α-tocopherol was also confirmed by small-angle X-ray diffraction analysis. The amount of hexagonal $H_{II}$ phase increased with increase in concentration of α-tocopherol. Egg PC dispersions containing a molar ratio of 0.8 of α-tocopherol gave a $^{31}$P-NMR spectrum of an approximately hexagonal $H_{II}$ type at 37 °C. The amount of hexagonal $H_{II}$ phase increased with increasing temperature, indicating that the α-tocopherol-induced phase transition is thermotropic and that the transition temperature of egg PC membranes from the lamellar to the hexagonal $H_{II}$ phase is lowered by α-tocopherol. The incorporation of α-tocopherol acetate did not induce any phase transition. This fact indicates that the hydroxyl group of α-tocopherol may play an important role in the hexagonal $H_{II}$ phase formation of egg PC dispersions.**

**Keywords** tocopherol; membrane; phase transition; $^{31}$P-NMR; hexagonal phase; bilayer phase; phosphatidylcholine; egg yolk phosphatidylcholine; small-angle X-ray diffraction

α-Tocopherol, which is the major component of vitamin E, is present in the membranes of intracellular organelles such as mitochondria and microsomes. In general, α-tocopherol is an antioxidant that inhibits the peroxidation of membrane lipids.

Using various physico-chemical techniques, numerous studies have been performed to investigate the effect of α-tocopherol on the structure, permeability and stability of model membranes, but there are large discrepancies in the results reported. For example, some reports have suggested an increase of permeability in the presence of α-tocopherol,[1-3] while others have indicated the depression of permeability by α-tocopherol.[4-6] These discrepancies could not be explained in terms of differences of experimental conditions. The effect of α-tocopherol on the membrane properties seems to be complex, and more detailed investigations are required.

The majority of the lipids in biomembranes are organized in a bilayer. However, vital membrane processes, such as membrane fusion[7] and protein insertion,[8,9] require the appearance of nonbilayer organization in biomembranes. In fact, some stable nonbilayer structures have been observed in biomembranes.[10-12] As nonbilayer structures, inverted micellar and hexagonal $H_{II}$ phase are most likely to be involved in membrane organization. Some compounds such as gramicidin,[13] myelin basic protein[14] and chlorpromazine[15] have been reported to cause hexagonal $H_{II}$ phase formation.

In this paper, using phosphate-31 nuclear magnetic resonance ($^{31}$P-NMR) and small-angle X-ray diffraction, we investigated whether or not α-tocopherol and its acetate can induce hexagonal $H_{II}$ phase formation in egg yolk phosphatidylcholine (egg PC) membranes.

## Materials and Methods

**Materials** Egg PC was purchased from Sigma Chemical Co. α-Tocopherol and α-tocopherol acetate were purchased from Tokyo Kasei Co., Ltd. Other reagents used were of analytical grade.

**Sample Preparation** Egg PC dispersions containing various amounts of α-tocopherol or its acetate were prepared as described in the literature.[6] Twenty-four milligrams of egg PC and the appropriate amounts of α-tocopherol or its acetate were combined in chloroform and the solvent was evaporated off under reduced pressure. Residual solvents were removed in a desiccator under vacuum for 2 h and the lipid films were dispersed in 3 ml of a 5 mM Tris–HCl buffer (pH 7.4) containing 0.2 mM ethylenediaminetetraacetic acid (EDTA) and 20% $D_2O$ by shaking on a Vortex mixer. The dispersions were transferred into NMR tubes (10 mm o.d.) under argon atmosphere and subjected to $^{31}$P-NMR spectroscopy.

**$^{31}$P-NMR Spectroscopy** Proton-decoupling $^{31}$P-NMR spectra were obtained at 109.32 MHz with a JEOL JNM-FX 270 spectrometer in the Fourier transform mode as described in the literature.[8] Accumulated free induction decays were obtaind with 5000 transients with an acquisition times of 1.0 s and a pulse width of 16 μs. Temperature was controlled with an accuracy of ±1 °C using a JEOL NM-PVTS2 unit. Chemical shift positions were measured relative to phosphoric acid. The chemical shift anisotropy of the bilayer signal was determined from the distance between the high and low field peaks in the spectrum. The relative amounts of bilayer, inverted micellar and hexagonal $H_{II}$ components in the $^{31}$P-NMR spectra were determined by computer subtraction of the pure bilayer component.

**Small-Angle X-Ray Diffraction** Samples prepared as described for the NMR measurements were loaded into capillary tubes of 1.5 mm diameter and 0.01 mm wall thickness. Small-angle X-ray diffraction measurements were performed on Rigaku RAD-RC using a rotating anode generator with a Cu$K_\alpha$ beam at 60 kV and 200 mA at 25 °C.

## Results

**$^{31}$P-NMR Measurements of Egg PC Dispersions** Figure 1 shows the $^{31}$P-NMR spectra of egg PC dispersions containing various amount of α-tocopherol or its acetate at 37 °C. The spectrum of pure egg PC dispersions showed a typical bilayer line shape with a high field peak and a low field shoulder, in agreement with the results of Cullis and de Kruijff.[16] The addition of a 0.2:1 molar ratio of α-tocopherol to egg PC dispersions caused no appreciable change in line shape of the $^{31}$P-NMR spectrum. At a 0.4:1 molar ratio of α-tocopherol to egg PC, a small peak appeared at a resonance position of 7 ppm. The intensity of the new signal increased with increasing α-tocopherol concentration. The new signal has reverse asymmetry compared to the bilayer spectra and is narrower by a factor of two, which is characteristic for lipids organized in a hexagonal $H_{II}$ phase. At a 0.8:1 molar ratio of α-tocopherol to egg PC, the spectrum indicated an approximately hexagonal $H_{II}$ phase formation in egg PC membranes. From

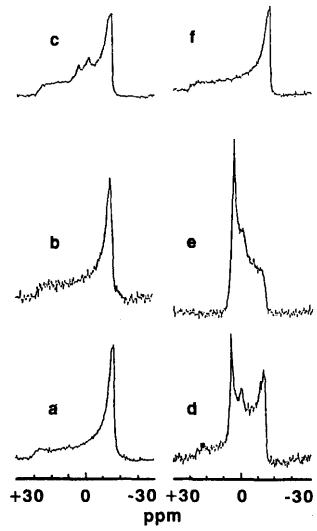


Fig. 1. Proton-Decoupling $^{31}$P-NMR Spectra of Aqueous Dispersions of Pure Egg PC and Egg PC Containing α-Tocopherol or Its Acetate at 37 °C

Egg PC dispersions (24 mg egg PC in 3 ml of a 5 mM Tris–HCl buffer containing 0.2 mM EDTA and 20% $D_2O$) were measured at 109.32 MHz in the Fourier-transform mode; 16 μs pulse width, 1.0 s acquisition time, and 5000 transients. (a) Pure egg PC, (b) α-tocopherol : egg PC molar ratio; 0.2 : 1, (c) 0.4 : 1, (d) 0.6 : 1, (e) 0.8 : 1, (f) tocopherol acetate : egg PC molar ratio; 0.8 : 1.

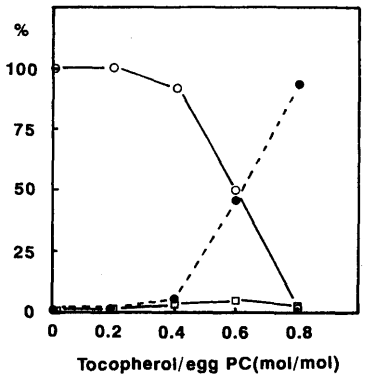


Fig. 2. Phase Diagram for Aqueous Dispersion of Egg PC Containing α-Tocopherol

Multilamellar bilayer phase (○), hexagonal $H_{II}$ phase (●), inverted micellar phase (□).

the $^{31}$P-NMR spectra, the phase diagram of egg PC–α-tocopherol dispersions was calculated. As shown in Fig. 2, in the egg PC dispersions with 0.6 : 1 molar ratio of α-tocopherol, one half of the phospholipid was organized in the hexagonal $H_{II}$ phase and the remainder was almost in a bilayer state. In addition, a small and rather broad isotropic component, which may be associated with intermediate structures such as lipidic particles and cubic phase, was observed over a range of α-tocopherol : egg PC molar ratio from 0.4 : 1 to 0.8 : 1 (Fig. 1).

Incorporation of α-tocopherol acetate, in which a hydroxyl group of the tocopherol chromanol nucleus is replaced by an acetate group, caused no change in the $^{31}$P-NMR spectrum up to the α-tocopherol : egg PC molar ratio of 0.8 : 1 (Fig. 1).

**Small-Angle X-Ray Diffraction Measurements** The phase transition in egg PC dispersions containing a molar ratio of 0.8 : 1 of α-tocopherol or its acetate was further investigated by small-angle X-ray diffraction at 25 °C.

Table I. Small-Angle X-Ray Diffraction Characteristics of Aqueous Dispersions of Egg PC and Egg PC Containing α-Tocopherol or Its Acetate

| Samples | Small-angle X-ray diffraction reflections (Å) | | |
|---|---|---|---|
| | 1 | $1/\sqrt{3}$ | 1/2 |
| Egg PC | 66.9 | n.d. | 33.1 |
| Egg PC/tocopherol (1/0.8) | 69.0 | 39.8 | 34.5 |
| Egg PC/tocopherol acetate (1/0.8) | 66.4 | n.d. | 32.5 |

The molar ratios of α-tocopherol or its acetate to egg PC are indicated in parenthesis.

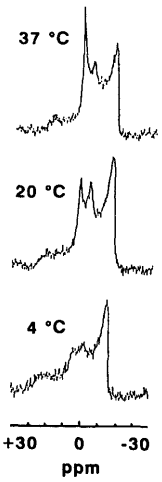


Fig. 3. Temperature Dependence of $^{31}$P-NMR Spectra for Aqueous Dispersions of Egg PC Containing α-Tocopherol

$^{31}$P-NMR spectra were measured as described in the legend of Fig. 1. α-Tocopherol : egg PC molar ratio; 0.6 : 1.

Table I lists the reflections of three different dispersions composed of pure egg PC, and egg PC containing α-tocopherol or its acetate. A pure egg PC dispersion showed two reflections, 66.9 and 33.1 Å. The egg PC containing α-tocopherol acetate showed similar reflections. A multilamellar organization of lipids shows a series of reflections with repeat distances related by $1 : 1/2 : 1/3, \cdots$ *etc.*, while a hexagonal $H_{II}$ organization of lipids shows additional reflections at distances related to the first-order reflection by $1/\sqrt{3}$, $1/\sqrt{7}, \cdots$ *etc.*, as shown by Luzzati.[17] The observation of repeat distances of 1 : 1/2 indicates that pure egg PC dispersions and those with α-tocopherol acetate have a lamellar organization. On the other hand, egg PC dispersions containing a 0.8 : 1 molar ratio of α-tocopherol showed an additional reflection, 39.8 Å, which is related as $1/\sqrt{3}$ to the repeat distance of the first-order reflection, indicating the occurrence of a lipid domain organized in a hexagonal $H_{II}$ phase. These findings were in agreement with the $^{31}$P-NMR results.

**Temperature Dependence of Phase Transition** $^{31}$P-NMR spectra of egg PC dispersions were measured over a range of α-tocopherol : egg PC molar ratio from 0.2 : 1 to 0.8 : 1. All dispersions showed temperature dependent change and it was found that the change of egg PC dispersions containing a molar ratio of 0.6 of α-tocopherol was most drastic, as demonstrated in Fig. 3. The $^{31}$P-NMR spectrum at 4 °C was an approximately bilayer signal. The bilayer type of $^{31}$P-NMR line shape decreased with increasing in temperature,

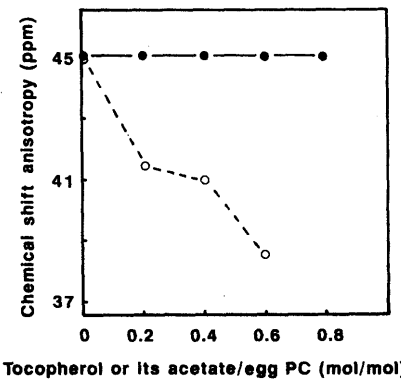


Fig. 4. Change of $^{31}P$ Chemical Shift Anisotropy of the Bilayer Signal in Egg PC Containing α-Tocopherol (○) or α-Tocopherol Acetate (●) at 37 °C

with concomitant appearance of a hexagonal $H_{II}$ signal. The $^{31}P$-NMR spectrum at 37 °C was a superposition of a bilayer signal and a hexagonal $H_{II}$ signal. This temperature-dependent behavior was reversible. The facts indicate that the α-tocopherol-induced phase transition is thermotropic.

**Change of $^{31}P$ Chemical Shift Anisotropy** Figure 4 shows the change of the chemical shift anisotropy of the bilayer signal in the $^{31}P$-NMR spectrum of egg PC dispersions containing various amounts of α-tocopherol or its acetate at 37 °C. The chemical shift anisotropy decreased from 45 ppm in the pure egg PC dispersions to 38.5 ppm in those containing a 0.6 : 1 molar ratio of α-tocopherol. The addition of α-tocopherol acetate to egg PC dispersions did not influence the chemical shift anisotropy, which was 45 ppm at all ratios tested. These findings indicate that the phosphate region of the polar head group in the lamellar phase is disturbed by the presence of α-tocopherol but not by α-tocopherol acetate.

## Discussion

In the present study, the effect of α-tocopherol on the phase structure of egg PC dispersions was demonstrated using $^{31}P$-NMR and small-angle X-ray diffraction. $^{31}P$-NMR spectra of egg PC dispersions containing α-tocopherol gave a typical hexagonal $H_{II}$ type signal, which has been confirmed by Cullis and de Kruijff,[16] and Seeling.[18] However, Thayer and Kohler[19] suggested that a particular conformation of phospholipid in a lamellar phase may give a hexagonal $H_{II}$ type signal. Further information on the structural characteristics was obtained by a small-angle X-ray diffraction study. The appearance of typical hexagonal $H_{II}$ reflections in the diffraction pattern also confirmed the formation of hexagonal $H_{II}$ phase by the addition of α-tocopherol.

The formation of hexagonal $H_{II}$ phase was observed on addition of α-tocopherol but not its acetate. This fact indicates that the hydroxyl group of α-tocopherol is important in this phase transition. It is generally assumed that α-tocopherol in lipid bilayers is oriented with the chromanol group towards the surface and with the hydrophobic phytol tail buried in the hydrocarbon region. Perly *et al.*[20] reported that the hydroxyl group of α-tocopherol is located near the phosphate moiety of the membrane surface. By comparing the carbon-13 nuclear magnetic resonance ($^{13}C$-NMR) spectra of α-tocopherol and its acetate in dipalmitoylphosphatidylcholine dispersions, Srivastava *et al.*[21] proposed that the hydroxyl group of α-tocopherol may be involved in a hydrogen bond with one of the oxygen atoms of the phospholipid. The dielectric constant obtained from the micropolarities shows hydrogen bond formation between the hydroxyl group of α-tocopherol and the ester carbonyl oxygen of PC.[22] Our results with $^{31}P$-NMR chemical shift anisotropy indicate that the phosphate region of the polar head group in egg PC dispersions is disturbed by the incorporation of α-tocopherol but not by that of its acetate. The hydrogen bond between the hydroxyl group of α-tocopherol and the ester carbonyl oxygen of egg PC should contribute to hexagonal $H_{II}$ phase formation in the dispersions.

The hexagonal $H_{II}$ phase in phospholipid dispersions is induced by not only α-tocopherol, but also several compounds such as gramicidin,[13] myelin basic protein[14] and chlorpromazine.[15] Several possibilities have been proposed concerning a determining factor for phase structure of phospholipid dispersions. Cullis *et al.*[16,23] suggested a relation of the phase structure to the shape of the phospholipid itself or the phospholipid-compound complex, that is, a cone-type shape favors hexagonal $H_{II}$ structure, while a cylindrical shape favors lamellar structure. The complex of egg PC with α-tocopherol, therefore, might form the shape of cone-type.

Another possibility is that the phase transition temperature from bilayer to hexagonal $H_{II}$ may be lowered by addition of an inducer of hexagonal $H_{II}$ phase. In the present study, the α-tocopherol-induced phase transition from bilayer to hexagonal $H_{II}$ phase is thermotropic. The incorporation of α-tocopherol has been reported to decrease the gel-liquid crystal phase transition temperature ($T_c$) for dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine, and dimyristoylphosphatidylethanolamine.[3,4,24] The $T_c$ for egg yolk phosphatidylethanolamine is at 19.3 °C, and the bilayer-hexagonal $H_{II}$ phase transition temperature ($T_{BH}$) for it is at 63 °C.[25] The $T_{BH}$ of egg PC is unknown, but it should be higher than the $T_c$, which was reported to be −7——15 °C.[26] Furthermore, Killian and de Kruijff[27] reported that the presence of gramicidin lowered the $T_{BH}$ for phosphatidylethanolamine dispersions. Similar to the effect of gramicidin, the $T_{BH}$ of egg PC may be lowered by incorporation of α-tocopherol, and hexagonal $H_{II}$ phase may be formed at 37 °C.

Cushley and Forrest[1] reported that incorporation of α-tocopherol into egg PC vesicles increases $Pr^{3+}$ permeability. In contrast, it was reported that incorporation of α-tocopherol into egg PC vesicles decreases the permeability to small molecules.[5,6] There was a large difference in the α-tocopherol contents between these studies. Therefore, the discrepancy might have arisen not only from the acyl chain composition of the phospholipids but also from the molar ratio of tocopherol in the membranes. The formation of hexagonal $H_{II}$ phase in the membranes demonstrated in the present study may be one of the causes of this discrepancy.

Lipid polymorphism is considered to be important in biological processes, such as membrane fusion, transbilayer movement of lipids and transport across membranes. It could be highly significant that α-tocopherol in the biomembranes induces a hexagonal $H_{II}$ phase.

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